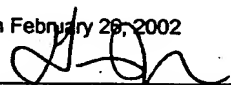


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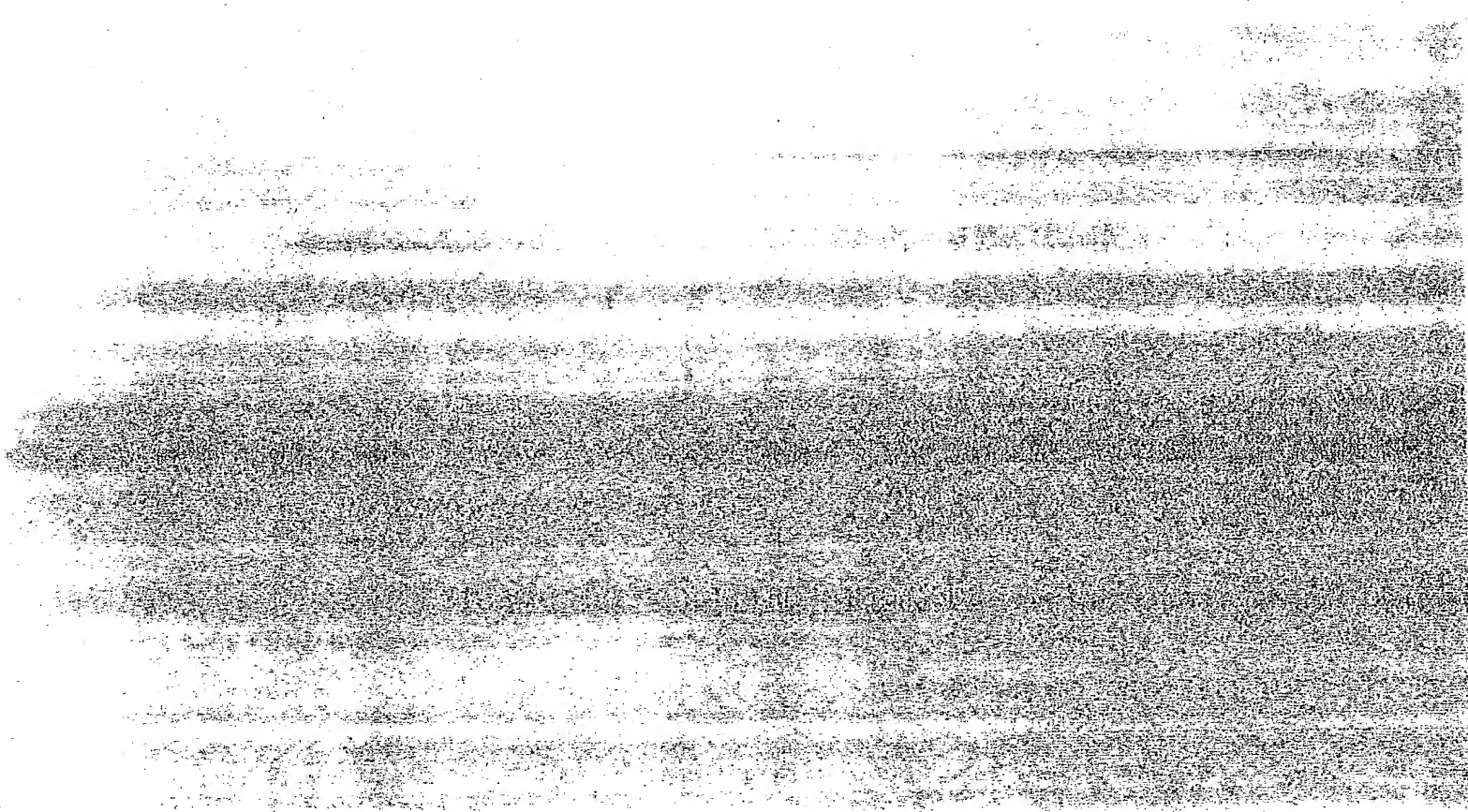


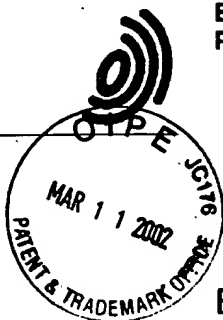
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Titre de l'invention:

Antibody heavy chain variable domains against human dietary enzymes, and their uses

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

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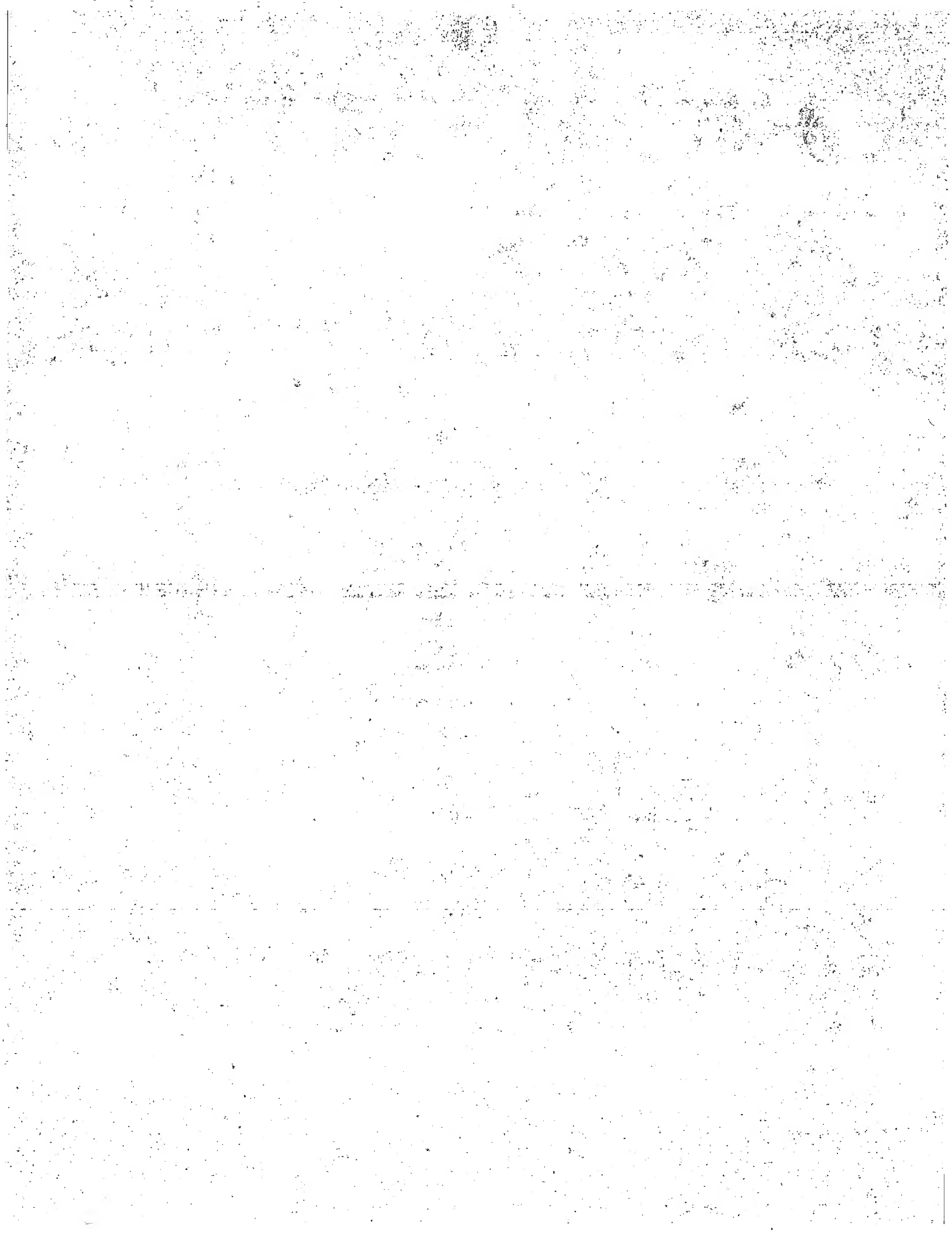
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Use of antibodies

Field of the Invention

5

The present invention is in the field of applied biotechnology and relates in particular to the inhibition of human lipase.

10 Background of the Invention

Cardiovascular diseases are the number one cause of death in the Western World. Epidemiologic and experimental data show clearly that high serum cholesterol levels, more
15 precise high level of Low Density Lipoprotein particles, which contain cholesterol show a strong correlation with the occurrence of cardiovascular diseases. It is also well known that foods products containing fats high in saturated fatty acids contribute to high serum Low Density
20 Lipoprotein levels. It has also be stated that hydrolysis of dietary fats, thereby liberating fatty acids in the stomach and intestinal tract increases the adsorption of cholesterol by the epithelial cells of the intestinal tract and consequently hydrolysis of dietary fats contribute to
25 increase of the serum Low Density Lipoprotein levels. Several human dietary enzymes are involved in this hydrolysis reaction. A further reason to reduce the hydrolysis of dietary fats and the subsequent liberation of fatty acids is to prevent or to reduce an increase of body
30 weight or event to reduce the body weight.

Also other enzymes in the gastrointestinal tract may be involved in undesirable physiological reactions.

Examples of such enzymes, which are referred to as human dietary enzymes include oxidoreductases, transferases, hydrolases (e.g. lipases, proteolytic enzymes and ureases), lyases, isomerases and ligases or synthetases.

5

There is therefore a need to find ways to reduce the amount of liberated fatty acids in the stomach and intestinal tract for example by inhibiting or modulating the activity of human dietary enzymes.

10

WO 98/34630 describes the use of a gastrointestinal lipase inhibitor for the manufacture of oral medicaments for treating or preventing type II diabetes mellitus. A preferred gastrointestinal lipase inhibitor is
15 tetrahydrolipstatin.

There is a desire to identify natural alternatives to tetrahydrolipostatin for the inhibition of human lipase or other human dietary enzymes. Even more desired is the
20 identification of materials which are capable of partial inhibition of human dietary enzymes, therewith possibly reducing, but not completely blocking the liberation of fatty acids in the human tract.

25 Aoubala et al in The Journal of Biological Chemistry, 8, 1995 pp 3932-3937 discloses monoclonal antibodies against human pancreatic lipase. Also Bezzine et al in Biochemistry (1998), 11846-11855 describes the binding of monoclonal antibodies to human pancreatic lipase. However a
30 problem with monoclonal antibodies is that they are expensive, difficult to prepare and are generally not stable under the conditions in the human tract.

There remains a continuing need for the development of new and improved methods for the inhibition or modulation of human dietary enzymes. In particular there is a need to develop effective gastrointestinal lipase inhibitors, which
5 can conveniently be prepared and which are sufficiently stable under the conditions found in the human tract.

Surprisingly it has been found that a special class of antibodies or fragments thereof namely those which are
10 naturally free of light chains and commonly referred to as V_H Hs can be used for the inhibition or modulation of human dietary enzymes.

Accordingly the present invention relates to an
15 antibody, or fragment thereof, capable of binding specifically to one or more human dietary enzymes, said antibody or fragment thereof comprising a heavy chain variable domain derived from an immunoglobulin naturally devoid of light chains, or a functional equivalent thereof.

20

Accordingly to a second aspect the invention relates to the use of an antibody, or fragment thereof, capable of binding specifically to one or more human dietary enzymes, said antibody or fragment thereof comprising a heavy chain
25 variable domain derived from an immunoglobulin naturally devoid of light chains, or a functional equivalent thereof for modulating the activity of human dietary enzymes.

In a third aspect the invention relates to the use of
30 an antibody, or fragment thereof, capable of binding specifically to one or more human dietary enzymes, said antibody or fragment thereof comprising a heavy chain variable domain derived from an immunoglobulin naturally

devoid of light chains, or a functional equivalent thereof in food products, including for example nutraceutical food products and dietary supplements.

5 In a fourth aspect the invention relates to the use of an antibody, or fragment thereof, capable of binding specifically to one or more human dietary enzymes, said antibody or fragment thereof comprising a heavy chain variable domain derived from an immunoglobulin naturally
10 devoid of light chains, or a functional equivalent thereof for the preparation of pharmaceutical products.

The invention will be further clarified in the following:

15

Brief description of Terms

The term " V_H " refers to the single heavy chain variable domain antibodies of the type that can be found in
20 Camelid mammals which are naturally devoid of light chains; synthetic V_H can be construed accordingly.

As used herein, the term "antibodies" refers to
25 immunoglobulins which may be derived from natural sources or may be synthetically produced, in whole or as antibody fragment.

An "antibody fragment" is a portion of a whole
30 antibody which retains the ability to exhibit antigen binding activity. Functionalized antibody fragments are also embraced in this term.

The term "functionalized antibody fragment" is used for indicating an antibody or fragment thereof to which one or more functional groups, including enzymes and other binding polypeptides, are attached resulting in fusion products of such antibody fragment with another biofunctional molecule.

The term "traditional antibody" is used for an antibody which normally consists of two heavy and two light chains or fragments thereof.

The term "human dietary enzymes" is used for enzymes which may be present and are physiologically active in the gastro-intestinal tract e.g. under stomach conditions or under intestinal conditions.

Detailed description of the invention

Antibodies are protein molecules belonging to a group of immunoglobulins generated by the immune system in response to an antigen. The structure of most antibody molecules is based on a unit comprising four polypeptides, two identical heavy chains and two identical light chains, which are covalently linked by disulphide bonds. Each of these chains is folded in discrete domains. The carboxy-terminal regions of both heavy and light chains are conserved in sequence and are called the constant regions, comprising one or more so-called C-domains. The amino-terminal regions of the heavy and light chains, also known as variable (V) domains, are variable in sequence and determine the specificity of the antibody. The regions in the variable domains of the light and heavy chains (V_L and V_H respectively) responsible for antigen binding activity

are known as the hypervariable or complementarity determining regions (CDR), while the framework regions (FR) are responsible for the typical immunoglobulin fold of the V-region.

5

Natural antibodies generally have at least two identical antigen-binding sites defined by the association of the heavy and light chain variable regions. Generally most naturally occurring antibodies need both a V_H and V_L to form a complete antigen binding site and retain full immunoreactivity.

10

More recently, immunoglobulins capable of exhibiting the functional properties of the four-chain immunoglobulins described above but which comprise two heavy polypeptide chains and which furthermore are devoid of light polypeptide chains have been described in WO 94/04678. Methods for the preparation of such antibodies or fragments thereof on a large scale comprising the transformation of a mould or yeast with an expressible DNA sequence encoding the antibody or fragment are described in WO 94/25591.

20

The immunoglobulins described in WO 94/4678, which may be isolated from the serum of Camelids, do not rely upon the association of heavy and light chain variable domains for the formation of the antigen-binding site but instead the heavy polypeptide chains alone naturally form the complete antigen binding site. These immunoglobulins, hereinafter referred to as "heavy-chain immunoglobulins" (or $V_{H\text{H}}$) are thus quite distinct from the heavy chains obtained by the degradation of common (four-chain) immunoglobulins or by direct cloning which thereby contain only a part of the antigen-binding site and require a light

30

chain partner for the formation of a complete antigen binding site in order to obtain optimal antigen-binding characteristics.

5 Surprisingly it has been found that V_HH 's, are capable of inhibiting human dietary enzymes, in particular enzymes involved in the hydrolysis of dietary fats, and thereby reduce the absorption of free fatty acids effectively.

10 It has been found that V_HH 's can be used for the inhibition of several human dietary enzymes. Examples of human dietary enzymes that can be inhibited are oxidoreductases, transferases, hydrolases (e.g. lipases, proteolytic enzymes and ureases), lyases, isomerases and
15 ligases or synthetases.

In a preferred embodiment of the invention V_HH 's are used for the inhibition of human enzymes involved in the hydrolysis of dietary fats, examples of these enzymes are
20 Human Pancreatic Lipase and Human Gastric Lipase.

Human Pancreatic Lipase (HPL) is the major lipase responsible for lipid conversion in adults, accounting for 48.5% of the hydrolysis of the triacylglyceride. The enzyme
25 is active at neutral pH in the small intestine, where it catalyses the hydrolysis of fatty acids in the sn-1 and sn-3 position of triacylglycerides. The enzyme requires a cofactor called colipase for lipolytic action on duodenal fats. The structure of HPL consists of an amino-terminal
30 domain (residues 1 through 336) and a carboxy-terminal domain (residues 337 through 448) that is involved in binding colipase.

Human Gastric Lipase (HGL) belongs to the family of the acid lipase family, which refers to its stability and activity in the highly acidic environment of the stomach. HGL is responsible for the hydrolysis of 17.5% of the meal triacylglyceride. The crystal structure of the enzyme, which contains 379 amino acid residues, reveals the presence of a core domain typical for the alpha/beta hydrolase family and a "cap" domain, similar to what has been found in Serine carboxypeptidases.

10

A preferred embodiment of the present invention involves the partial inhibition of human dietary enzymes using V_HH 's. Preferably the enzymes, for example the human lipases, are only partially inhibited to ensure that no deficiencies of important ingredients will occur. Preferably the level of inhibition, measured in accordance to Figure 3 is between 2 to 90%, more preferred 3-30%, most preferred 5-20%.

20

For the purpose of the invention antibodies can be used in their entirety (e.g. in a form which is equal to or closely resembles the natural form in the Camelid source). Alternatively, however fragments of these antibodies e.g. V_HH 's may be used. If fragments are used then it is preferred that these fragments comprise one or more sequences which are equal to or closely resemble the CDR regions in the natural V_HH 's. Particularly preferably these fragments comprise a sequence which is equal to or closely resembles the CDR3 region of a natural V_HH .

30

In a particular preferred embodiment the V_HH 's (including either entire V_HH 's or fragments thereof)

according to the present invention are characterised by a CDR3 selected from the following classes:

- 5 (I) ARSLX₁X₂TPTSVDY
(II) RGGLTQYSEHDY
(III) TGAEGHY
(IV) TDMGRYGTSEW

10 Wherein X₁ is V or E and X₂ is Q or L.

Preferred examples of V_HH's of the first class are HGL#1 and HGL#16. Preferred examples of V_HH's of the second class are HGL#4 and HGL#10. Preferred examples of V_HH's of
15 the third class are HGL#8 and HGL#9. A preferred example of V_HH's of the fourth class is HGL#11.

In another particular preferred embodiment the V_HH's according to the present invention are characterised by a
20 CDR3 selected from the following classes:

- (a) DVRPYRTSRYLEX₃
(b) QVRVRFSSDYTNV
(c) LIRRKFTSEYNEY
25 (d) LITRWDKSVNDY
(e) RRSNYDRSWGDI
(f) LISSYDGSWNDY
(g) HITPAGSSNYVYGY
(h) DIRKFTSGYSHY

30

Whereby X₃ is V or L or I

An example of a $V_{\text{H}}\text{H}$ of class (a) is HPL#12, HPL#14 and HPL#30

An example of a $V_{\text{H}}\text{H}$ of class (b) is HPL#19

An example of a $V_{\text{H}}\text{H}$ of class (c) is HPL#18

5 An example of a $V_{\text{H}}\text{H}$ of class (d) is HPL#13

An example of a $V_{\text{H}}\text{H}$ of class (e) is HPL#11

An example of a $V_{\text{H}}\text{H}$ of class (f) is HPL#22

An example of a $V_{\text{H}}\text{H}$ of class (g) is HPL#15

An example of a $V_{\text{H}}\text{H}$ of class (h) is HPL#17

10

$V_{\text{H}}\text{H}$'s in accordance to the present invention can be used for the inhibition of the activity of human dietary enzymes. Surprisingly it has been found that the $V_{\text{H}}\text{H}$'s are often more stable than traditional antibodies under
15 conditions similar to those in the gastric intestinal tract. In particular preferred $V_{\text{H}}\text{H}$'s in accordance to the invention have a stability (as measured in example 4.3) of at least 75% after 1 hour.

20 $V_{\text{H}}\text{H}$'s in accordance to the present invention can be administered to human beings in any desirable form. In a first preferred embodiment of the invention the $V_{\text{H}}\text{H}$'s can be used in pharmaceutical compositions. These compositions normally comprise in addition to the $V_{\text{H}}\text{H}$'s a suitable
25 carrier material. For example the $V_{\text{H}}\text{H}$'s can be incorporated into medicines for oral use such as tablets, capsules, medicinal liquors, powders, but other application forms e.g. as an injection, topical applications etc may equally be suitable.

30

In a second preferred embodiment of the inventions the $V_{\text{H}}\text{H}$'s can be used in food products. Examples of suitable food products are margarine and other bread spreads,

dressings, beverages including fruit juices and tea and coffee, bakery products such as cookies, biscuits, bread, pizza etc, sauces including hot or cold sauces, frozen confectionery materials e.f water-ice or ice-cream, dairy
5 products e.g. desserts, yoghurt, cheese etc, cereal products, for example breakfast cereals, sweets such as pastilles, lollypops, bars, chocolate etc.

Typically a suitable intake per meal of antibodies
10 could be such that the molar ratio of antibody to the relevant dietary enzyme is between 10 : 1 and 1 : 10. It is well within the ability of the skilled person to adapt the concentration of antibodies in the product such that these amounts are consumed.

15

The present invention may be more fully understood with reference to the following description when read together with the accompanying drawings in which:

20 Figure 1 shows the titration of serum antibodies from the llama immunised with Human Pancreatic Lipase in ELISA on enzyme recognition (A) and on inhibition of lipase activity (B);

25 Figure 2 analyses the efficiency of individual $V_{H}H$ fragments to recognise Human Pancreatic Lipase (determined with ELISA) (2A) and to inhibit lipase activity (2B);

30 Figure 3 shows the titration of serum antibodies of the llama immunised with Human Gastric Lipase on enzyme recognition (3A), and on lipase inhibition (3B).

Figure 4 is a restriction map of phagemid pUR5071.

The invention is applicable to the use of any immunoglobulin variable domain, which forms a complete antigen binding site. The immunoglobulin may be derived from natural sources or synthetically produced. Preferably, the invention relates to the use of heavy chain variable domains derived from an immunoglobulin devoid of light chains, most suitably from an immunoglobulin naturally devoid of light chains such as are obtainable from lymphoid cells, especially peripheral blood lymphocytes, bone marrow cells or spleen cells derived from Camelids as described in WO 94/04678 (Casterman et al).

It will be appreciated that heavy chain variable domains derived from other immunoglobulins modified to enable them to function as monovalent binding domains in the same way as the heavy chain variable domains derived from Camelids may also suitably be used according to the invention. For the purpose of this invention these molecules are referred to as functional equivalents.

A major advantage of the use of single domain binding units, which are heavy chain variable domains derived from Camelids, is their unusual stability against extreme pH, degradation by proteases, high concentrations of salts and high temperatures, which makes these fragments suitable for application in food products and to be effective in the Gastro-intestinal tract. Another benefit of single domain binding units is that these molecules can readily and conveniently be produced economically on a large scale, for example using a transformed lower eukaryotic host as described in WO 94/25591 (Unilever). This describes a

production system that delivers high amounts of secreted antibody fragments with a low degree of impurities present in the secreted fraction, thereby enabling simple downstream processing procedures for purification.

5

The invention also provides host cells and expression vectors enabling high levels of production and secretion of the binding proteins.

10 Heavy chain variable domains derived from an immunoglobulin naturally devoid of light chains having a determined antigen specificity may conveniently be obtained by screening expression libraries of cloned fragments of genes encoding Camelid immunoglobulins generated using
15 conventional techniques, as described, for example, in EP-A-0584421 and Example 1. Preferred methods to enrich for binding domains recognising the human dietary enzyme, thereby limiting the numbers of clones, which have to be screened for the identification of inhibiting fragments,
20 are yeast display (WO 94/01567 from Unilever) or phage display.

Enzyme inhibiting antigen binding proteins may be prepared by transforming a host by incorporating a gene
25 encoding the polypeptide as set forth above and expressing said gene in said host.

Suitably the host or hosts may be selected from prokaryotic bacteria, such as Gram-negative bacteria, for
30 example *Escherichia coli*, and Gram-positive bacteria, for example *Bacillus subtilis* and in particular lactic acid bacteria, lower eukaryotes such as yeasts, for example belonging to the genera *Saccharomyces*, *Kluyveromyces*,

Hansenula or *Pichia*, or moulds such as those belonging to the genera *Aspergillus* or *Trichoderma*.

Preferred hosts for use in connection with the present invention are the lower eukaryotic moulds and yeasts.

Techniques for synthesising genes, incorporating them into hosts and expressing genes in hosts are well known in the art and the skilled person would readily be able to put the invention into effect using common general knowledge.

Proteins for use according to the invention may be recovered and purified using conventional techniques such as affinity chromatography, ion exchange chromatography or gel filtration chromatography.

The binding activity of the binding proteins prepared according to the invention may conveniently be measured by standard techniques known in the art such as enzyme-linked immunoadsorbant assay (ELISA), radioimmune assay (RIA) or with biosensors.

The following examples are provided by way of illustration only. Techniques used for the manipulation and analysis of nucleic acid materials were performed as described in (Sambrook et al., 1990), unless otherwise indicated.

30 EXAMPLES

EXAMPLE 1. Induction of a humoral immune response in llama.

Human Pancreatic Lipase (HPL) was purified as described by De Caro, A., Figarella, C., Amic, J., Michel, R. & Guy, O. (1977). *Biochim. Biophys. Acta* 490(2), 411-5 419.

A llama was immunised with an HPL in oil emulsion obtained by mixing 2 ml antigen in water and 3 ml Specol, (Bokhout, B.A., Van Gaalen, C. & Van der Heijden, P.J. 10 (1981) *Vet. Immunol. Immunopath.* 2, 491-500.

Per immunisation 4 times 1.25 ml water in oil emulsion was injected said 1.25 ml containing 200 µg enzyme. Each immunisation involved 4 injections two of the injections 15 were subcutaneous, the other two inter-muscular. The second immunisation was performed four weeks after the first injection, the third immunisation 8 weeks after the first injection and the fourth immunisation 12 weeks after the first injection. The immune response was monitored by 20 titration of serum samples in two different assays. In the first assay the serum antibodies recognising HPL were quantified in ELISA (Fig. 1A), and in the other one the titre of inhibiting antibodies was determined in an enzyme activity assay (Fig. 1B).

25

For ELISA *in vitro* biotinylated HPL (prepared as described in paragraph 2.2) was immobilised indirectly via streptavidin. Streptavidin was coated at 5 µg/ml in Phosphate Buffered Saline (PBS) during two hours at room 30 temperature in maxi-sorb plates (NUNC). The coat solution was removed and, after washing with 0.05 vol% Tween-20 in PBS (PBST), the wells were blocked during 30 minutes at room

temperature with a 4 wt% skimmed milk solution made in PBS. Biotinylated HPL was captured by the coated streptavidin during 16 hours at 4°C from a solution with a concentration of 2.5 µg/ml enzyme in PBST, followed by washing of the 5 plate with PBTS to remove free biotinylated HPL.

Serum samples were tested in serial dilutions (in 2% skimmed milk solution in PBST). Subsequently the bound llama antibodies were detected with polyclonal rabbit-anti-
10 llama antiserum (obtained via immunising rabbits with llama immunoglobulins purified via Protein A and Protein G columns (Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman, N. & Hamers, R. (1993). *Nature* 363(6428), 446-448.) and swine-
15 anti-rabbit immunoglobulins (DAKO) conjugated to horse radish peroxidase. Finally the peroxidase enzyme-activity was determined with tetramethyl-benzidine and ureaperoxide as substrates and the optical density was measured at 450 nm after termination of the reaction by the addition of
20 H₂SO₄.

The titre of inhibiting antibodies was determined in the LIPASE-PS assay (Sigma Diagnostics), in which the enzymatic hydrolysis of 1,2-diglyceride into 2-
25 monoglyceride and fatty acid can be measured kinetically in a spectrophotometer at a wavelength of 550 nm. For this assay 5 µl (diluted) serum was mixed with 10 µl distilled water and 5 µl HPL (approximately 250 lipase units/ml. From this mixture 5 µl was added to 150 µl substrate solution
30 (LIPASE-PS Substrate Reagent) using the wells of a microtiter plate as reaction vessels. After incubating the plate for 8 minutes at 37°C, Activator solution (50

μ l/well) was added and colour development was measured kinetically during a period of 10 minutes at 550 nm and 37°C, whereby a change in colour intensity implied enzymatic activity.

5

EXAMPLE 2. Cloning, selection and screening of clones producing llama V_HH fragments inhibiting Human Pancreatic Lipase.

5

2.1 Isolation of V_HH fragments against Human Pancreatic Lipase.

Llama RNA was isolated from its lymphocytes using a blood sample taken 8 weeks after the first immunisation. At that point in time the llama had the highest titre of HPL recognising antibodies as measured in ELISA.

A blood sample of about 150 ml was taken and an enriched lymphocyte population was obtained via centrifugation on a Ficoll Paque (Pharmacia) discontinuous gradient. From these cells total RNA was isolated by guanidium thiocyanate extraction according to Chomczynski, P. & Sacchi, N. (1987). *Anal. Biochem.* 162(1), 156-159.

After first strand cDNA synthesis using MMLV-RT (Gibco-BRL) and random oligonucleotide primers (Pharmacia), DNA fragments encoding V_HH and part of the long or short hinge region were amplified by PCR using three specific primers as described in example II.2.1 of WO99/46300.

25

The DNA-fragments generated by PCR were digested with *Pst*I (coinciding with codon 4 and 5 of the V_HH domain, encoding the amino acids L-Q) and *Not*I (introduced at the 5' end of the hinge specific oligonucleotide primers, coinciding with the amino acid sequence A-A-A). The digested PCR products were cloned in the phagemid vector pUR5071 (figure 4) as gene-fragments encoding the V_HH domain including the hinge region fused to the gene III

protein of the *E. coli* bacteriophage M13. A first display library with 1.5×10^7 clones containing the short hinge derived V_HH fragments and a second library of 6.2×10^7 clones with long hinge derived V_HH , was constructed in 5 phagemid vector pUR5071.

2.2 Enrichment of HPL binding V_HH domains via phage display methodology.

- 10 Phage particles exposing V_HH fragments were prepared by infection of *E. coli* cells harbouring the phagemid with helperphage VCS-M13 according to Marks, J.D et.al (1991) *J. Mol. Biol.* 222, 581-597.
- 15 Free V_HH fragments were removed by precipitation of phage from the culture supernatant with PEG6000, thereby avoiding a disturbing competition between phage bound and free v_HH fragments. "In solution" capture of *E. coli* phage exposing HPL specific antibody fragments was performed with
- 20 in vitro biotinylated lipase (EZ link NHS-biotin) covalently coupled to free NH_2 -groups of the lipase according to the instructions of the supplier; the molar ratio between biotin and lipase was 15 to 1). For selection
- 15 nM and 40 nM Human Pancreatic Lipase was used in round
- 25 one and 0.6 nM and 3 nM in round two. The carboxy terminal domain was prepared by proteolysis with chymotrypsin and purified with reversed phase HPLC. Lipase was biotinylated and used for selection of the immune library (combined short hinge and long hinge) at 15 and 70 nM during round
- 30 one and at 1 and 3 and 15 nM during round two. During the binding phase of the selection "application conditions" (inclusion of 5.3 mM cholic acid and 36 mM deoxycholate) were used. Phage particles bound via their displayed

antibody fragments to the biotinylated lipase or the carboxy terminal domain peptide were pulled out of the solution with streptavidin coated magnetic beads (Dyna) (see (Hawkins, T, DNA Seq. 1992; 3(2) 65-9). After washing, 5 phage was eluted with triethylamine.

Individual *E. coli* clones obtained after two rounds of selection were grown in wells of microtiter plates, and the production of $V_{\text{H}}\text{H}$ fragments was induced by the addition of 10 0.1 mM isopropyl- β -D-thiogalactopyranoside. After 16 hours of growth, the culture supernatant of the clones was analysed in ELISA for the presence of $V_{\text{H}}\text{H}$ fragments, which specifically bind to indirectly immobilised biotinylated HPL, using a streptavidin coated plate as a negative 15 control. Bound $V_{\text{H}}\text{H}$ fragments were detected with rabbit anti-llama $V_{\text{H}}\text{H}$ polyclonal antibodies followed by incubation with goat anti-rabbit polyclonal antibodies conjugated to horse radish peroxidase (BIORAD), or with mouse monoclonal anti-myc antibody ATCC myc 1-9E 10-2 followed by incubation 20 with polyclonal rabbit anti-mouse conjugated to horse radish peroxidase (DAKO). The myc-tag is encoded in the phage display vector, which results in the addition of this peptide sequence to the carboxy terminus of the $V_{\text{H}}\text{H}$ fragments.

25

2.3 Development of a high-throughput screening assay for the identification of lipase inhibiting $V_{\text{H}}\text{H}$ fragments.

The lipase inhibiting capacity of the $V_{\text{H}}\text{H}$ fragments 30 was demonstrated in an enzyme activity assay (Sigma). Different anti-HPL clones were identified by their characteristic *Hin*FI fingerprint pattern (Marks et al., as above), for which the $V_{\text{H}}\text{H}$ encoding insert was amplified

with the M13REV- and the gene III-primer. The resulting PCR-product was digested with the restriction enzyme *HinFI*, whose recognition site frequently occurs within antibody genes. The representative clones were grown on 5 ml scale and the cells were harvested after a relative short induction time of 3.5 hours at 37°C. An osmotic shock was given by resuspending and incubating the pelleted cells in 0.5 ml of ice-cold PBS during two to sixteen hours at 4°C. Spheroplasts were removed by centrifugation and the supernatant, containing the periplasmic proteins, was tested in ELISA in serial dilutions for binding to biotinylated HPL and in the lipase enzyme assay for their capacity to inhibit the enzyme.

Selection with biotinylated lipase or its carboxy terminal domain resulted in the isolation of clones, which produce inhibiting V_HH fragments.

2.4 Sequences of HPL inhibiting V_HH fragments.

By using biotinylated HPL enzyme 190 inhibiting V_HH fragments were selected, 8 of these were sequenced, these fragments are coded HPL#11, HPL#12, HPL#13, HPL#15, HPL#18 and HPL#19

By using the carboxy terminal domain of HPL 95 lipase inhibiting V_HH fragment were selected, 6 were sequenced, resulting in one new class represented by HPL#22.

With respect to the length of CDR3, which is the most important region for binding to the antigen, the antibodies can be grouped in three classes, as is shown in the

following amino acid sequences, wherein the respective CDR regions are indicated in bold CDR1 being the first bold strand etc. HPL#12, HPL#18 and HPL#19 are characterised by a CDR3 region having a length of 13 amino acids, HPL#11, 5 HPL#13 and HPL#22 are characterised by a CDR3 region of 12 amino acids and HPL#15 is characterised by a CDR3 region of 14 amino acids.

HPL#11

10 QVQLQDSGGGLVQAGGSLRLSCAASGSIFS **SDIMG** WYRQAPGKEREAVA 49
RITRGGTTSYADSVK GRFTISRDNKNTMYLQMNSLKPEDTAVYYCNA 97
RRSN--YDRSWG DY WGQGTQVTVSS AHHSEDPSS 129

15 HPL#12

QVQLQESGGGLVQAGGSLRLSCAASGSIGS **IHTMG** WYRQTPGKERDVVA 49
TIQDGGSTNYADSVK GRFTISRDNLTNTVYLMNDLKPEDTAVYYCNA 97
DVRP--YRTSRYLEV WGQGTQVTVSS EPKTPKPQP 130

20 HPL#13

QVQLQESGGGLVQAGGSLRLSCAASGTILS **IIYMD** WYRQTPGKQRELVA 49
RITAGGSTNYADSAK GRFTISKDNKNTVYLMNSLKPEDTAVYYCNA 97
LITR--WDKSVNDY WGQGTQVTVSS EPKTPKPQP 129

25

HPL#14

QVQLQESGGGLVQAGGSLRLSCAASGSIGS **IHTMG** WYRQTPGTERDVVA
TIQDGGSTNYADSVK GRFTISRDNILNTVYLMNSLKPEDTAVYHCNA

30 **DVRPYRTSRYLEL** WGQGTQVTVSS EPKTPKPQP

HPL#15

QVQLQESGGGLVQAGGSLRLSCAASGSISS **INVMG** WFRQAPGKQRELVA 49
SITSGGSTNYADSLK GRFTISRDNKNAVYLMNNLKPEDTAVYYCNA 97

35 **HITPAGSSNYVYGY** WGHGTVTVSS EPKTPKPQP 131

HPL#18

QVQLQDSGGGLVQAGGSLRLSCAASGTIGD **IYTMA** WHRQAPGKERELVA 49
SATESGSPNYADPVK GRFTISRDNGLKLTIVYLQMNSLKPEDTAVYYCNA 97
5 **LIRR-KFTSEYNEY** WGQGTQVTVSS EPKTPKPQP 130

HPL#19

QVQLQDSGGGLVQTGGSLRLSCAASGPIGD **VYLMG** WYRQAPGKQREMVA 49
10 **SITATGPPNYTDSVK** GRFTISRDNNDKNTTEYLQMNNLKPEDTAVYYCNA 97
QVRV-RFSSDYTNY WGQGTQVTVSS EPKTPKPQP 130

HPL#22

QVQLQESGGGLVQAGGSLRLSCAASGSIRS **ISIMT** WYRQAPGKERELVA 49
15 **RMSSDGTTSYTDSMK** GRFTISRDNNAKNTVYLHMNNLKPEDTAVYYCKA 97
LISS--YDGSWNDY GGQGTQVTVSS EPKTPKPQP 129

HPL#30

20 QVQLQDSGGGLVQAGGSLRLSCAASGSIGD **IHTMG** WYRQTPGKQRDVV
ATIQSGGSTNYADSVK GRFTISRDNLTNTVYLQMNDLKPEDTGVIYYWNA
DVRPYRTSRYLEI WGQGTLVTVFL EPKTPKPQP.

EXAMPLE 3. The efficacy of V_HH fragments to inhibit Human Pancreatic Lipase.

3.1 Recloning in episomal plasmid system for production of anti-HPL V_HH fragments in *Saccharomyces cerevisiae*

The V_HH encoding sequences of clones HPL#11, HPL#13, HPL#15, HPL#17, HPL#18 and HPL#19 were digested with *Pst*I and *Bst*EII from the *E. coli* phagemid vectors pUR5084, pUR5082, pUR5095, pUR5080, pUR5086 and pUR5087 respectively, and cloned in the episomal *S. cerevisiae* plasmid pUR4547 (deposited at the CBS, Baarn, The Netherlands as CBS100012) for the secretion of V_HH fragments, thereby obtaining pUR5091, pUR5090, pUR1403, pUR5088, pUR5092 and pUR5093 respectively. Secretion of V_HH fragments with carboxy terminal tag-sequences was accomplished by cloning in plasmid pUR4585, which is identical to plasmid pUR4547 except encoding the myc-tag for detection with monoclonal antibody myc 1 - 9E10.2 (ATCC) and the hexahistidine tail for purification with IMAC. Plasmid constructs pUR5099, pUR5098, pUR5097, pUR5096, pUR5263 and pUR5264 were made encoding the tagged V_HH fragments of HPL#11, HPL#13, HPL#15, HPL#17, HPL#18 and HPL#19 respectively.

25

Both parental plasmids pUR4547 and pUR4585 contain the GAL7-promoter for inducible expression of the V_HH gene product, the selectable markers bla (β -lactamase) to discriminate transformants in *E. coli* by resistance to the antibiotic ampicillin and *Leu2d* (β -isopropylmalate dehydrogenase) for selection of transformed *S. cerevisiae*, and an *E. coli* origin of replication. Secretion is

accomplished by fusing the *SUC2* leader sequence to the amino terminus of the V_H H product according to Harmsen, M.M. et al (1993) Gene 125, 115-123.

5 Clones HPL#14 and HPL#16, which lack the *Bst*EII-site, were cloned as *Pst*I/*Not*I-fragments (including their hinge region) in secretion plasmid pUR1400, which is identical to pUR4585 except with the additional *Not*I cloning site situated between the *Bst*EII-site and the myc-
10 /hexahistidine-tags. In this way plasmid constructs pUR5265 and pUR5266 were obtained containing the V_H H genes of clones HPL#14 and HPL#16 respectively.

Transformants in *E. coli* containing the *S. cerevisiae*
15 shuttle constructs with the V_H H genes were identified by PCR screening using primers M13REV and M13U for amplification of the V_H H encoding insert and by restriction enzyme analysis on plasmid DNA. Plasmid DNA purified with the Quick-prep kit (Qiagen) was used for
20 transformation of *S. cerevisiae* strain VWK18gal1::URA3, *ura3, leu2* by the lithium acetate procedure as described by Gietz, R.D et al (1995) Yeast 11(4), 355-360.

Two clones from each construct were grown for 24 hours
25 at 30°C in YNB medium (0.67 % Yeast Nitrogen without amino acids (Difio)) containing 2 % glucose. For V_H H gene expression both pre-cultures were diluted 1/10 in 1 ml of YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% galactose) for induction of the GAL7 promotor and grown
30 during 48 hours at 30°C using 8 wells culture plates for cultivation. The V_H H production in the medium fraction of these clones was examined by analysis on a Coomassie blue stained polyacrylamide gel. Their functional

characteristics were confirmed in ELISA on indirectly immobilised biotinylated HPL and in the lipase enzyme activity assay.

5 3.2 Purification and characterisation of V_HH fragments produced by *S. cerevisiae*.

After confirming the binding and inhibitory characteristics observed before with the *E. coli* produced V_HH fragments, the *S. cerevisiae* transformants were induced in 250 ml shake flasks using 30 ml of culture medium as described in section 3.1. Following 48 hours of induction, the medium fraction was separated from the cells by centrifugation. For purification via immobilised metal affinity chromatography (IMAC) 12 ml of each medium fraction was adjusted to 50 mM NaH₂PO₄ (pH 8.0), 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl and added to 1 ml of TALON column material (CLONTECH). The hexahistidine tagged V_HH fragments were bound to the immobilised metal ions in a batchwise fashion by rotating the column material head-over-head during 30 minutes; washing of the column material with sonication buffer and elution with 100 mM imidazol (Sigma) was performed according to the instructions of the supplier (CLONTECH). After removal of imidazol by dialysis against PBS, the amount of purified V_HH was determined by measuring the optical density at 280 nm using the calculated molar extinction coefficient. Analysis on a Coomassie stained protein gel confirmed the purity and the measured amount of V_HH. Between 100 and 500 µg antibody fragment was purified from 12 ml of culture.

The results of the ELISA measurements are given in Figure 2A.

3.3 Measurement of inhibition in intestinal juices.

Yeast comprising pUR5099, pUR5097, pUR5263 and pUR5264
5 encoding the V_HH domains of HPL#11, HPL#15, HPL#18 and
HPL#19 respectively were cultivated and induced in one
litre of YPD medium. The cells were removed by
centrifugation and the medium fraction containing the
antibody fragment was concentrated five-fold with a
10 dialysis unit (Hemophan fiber dialyzer GFSplus 12, Gambro,
Breda). HPL#11, HPL#15 and HPL#18 were purified by affinity
chromatography on protein A sepharose (Pharmacia).

HPL#19, which did not bind to protein A, was purified
15 with IMAC, yielding 3.7 mg V_HH per litre of culture. After
dialysis against PBS, the fractions can be used for
inhibition experiments in intestinal juice.

EXAMPLE 4. Isolation of llama V_HH fragments capable to inhibit Human Gastric Lipase.

4.1 Isolation and production of inhibiting V_HH fragments against Human Gastric Lipase.

A male llama was immunised with Human Gastric Lipase purified as described in Moreau, H et al (1992) *J. Mol. Biol.* 225(1), 147-153 according to the procedure indicated above and the titration of blood samples was performed on biotinylated enzyme in ELISA as described in example 1 (Fig. 3A).

The titre of inhibiting antibodies was determined in an enzyme assay, in which 1,2-0-dilauryl-rac-glycero-3-glutaric acid-resorufin ester (DGGR, Boehringer Mannheim) was used as chromogenic substrate. For the assay 5 µl HGL solution (0.1 mg/ml) was pre-incubated with 10 µl of (diluted) serum sample in the well of a microtiter plate. The reaction was started by the addition of 165 µl of buffer containing 100 mM MES pH6.0 and 0.6 M NaCl and 20 µl DGGR solution (1 mg/ml in dioxane/Thesit-solution (1:1)). The kinetics of the enzymatic conversion was measured at 572 nm during a period of 30 minutes at 37 °C with a Spectramax spectrophotometer. With this assay the titre of enzyme inhibiting antibodies was determined in serum samples taken after different time intervals (Fig. 3B).

RNA was isolated from the lymphocytes of a blood sample taken 9 weeks after the start of the immunisation. Random primed cDNA was prepared and used for the amplification of short hinge and long hinge derived V_HH

fragments, which were cloned in the phagemid vector pUR5071. A short hinge derived library was constructed, which contains 5.4×10^7 clones and a long hinge derived library with 4.5×10^7 clones.

5

Selections were performed with 20 and 60 nM of biotinylated Human Gastric Lipase at the first round and subsequently with 1 or 6 nM lipase during the second round according to the method described in paragraph 2.2. During 10 round one physiological conditions (PBS) were used, while at round two the conditions of the stomach were imitated by lowering the pH to 4.5 with 25 mM sodium acetate buffer and inclusion of proteases pepsin. In this way acid and protease resistant antibody fragments were retrieved from 15 the library.

Culture supernatants from individual clones grown and induced in microtiter plates were analysed in ELISA on indirectly immobilised HGL and in the enzyme activity 20 assay. Approximately 30 to 60% of the enzyme recognising antibody fragments showed to inhibit the enzyme.

Sequences of 8 gene segments are given below, whereby the CDR regions are indicated in bold. The V_H encoding 25 gene segments could be classified into four groups according to the length of their CDR3 (see below). The four groups are: HGL#1 and HGL#16; HGL#4 and HGL#10; HGL#8, HGL#9 and HGL#15; and HGL#11.

HGL#1
QVQLQESGGGLVQAGGSLRLSCAASGFDPR **YNTMA** WYRQAPGKQRELVA 49
TIASTYRTSYADSVK GRFTISRDNARGTVYLQMNSLKPEDTAVYYCAA 97
ARSLVQTPTSIDY WGQGTQVTVSS AHHSEDPSS 130

5
HGL#4
QVQLQESGGGLVQAGGSLRLSCAASGSTFS **FNAMG** WYRQVPGKQRELVA 49
AIGNDGATYYVDSVK GRFTIARENAKNTVYLQMSSLKPEDTAVYYCKG 97
RGGLTQYSEHDY WGQGTQVTVSS EPKTPKPQP 129

10
HGL#8
QVQLQESGGGLVQTGGSLRLSCAASGSIGS **MYVLS** WYRQAPGKQREPVA 49
ALMGSGSTTYADSVK GRFTISRDNINKNTMYLQMNSLTPEDTGVYYCAG 97
TGAEGHY WGQGTQVTVSS AHHSEDPSS 124

15
HGL#9
QVQLQESGGGLVQAGGSLRLSCAASGSIGS **LYVMS** WYRQAPGKQREPVA 49
ALMGSGSTTYADSVK GRFTISRDNINKNTMYLQMNSLKPEDTGVYYCAG 97
TGAEGHY WGQGTQVTVSS EPKTPKPQP 124

20
HGL#10
QVQLQESGGDLVQAGGSLRLACAASGSTFS **FNAMG** WYRQVPGKQRELVA 49
AIGNDGSTYYVNSVK GRFTISRENAKNTVYLQMNSLKPEDTAVYYCKG 97
RGGLTQYSEHDY WGQGTQVTVSS EPKTPKPQP 129

25
HGL#11
QVQLQESGGGLVQAGGSLRLSCTASGTTDN **INAMG** WYRQAPGKQRELVA 49
AISSGGDTYYTEFVK GRFTISRDNNAKAVYLMNNLKSEDTAVYSCKM 97
TDMGRYGTSEW WGQGTQVTVSS EPKTPKPQP 128

30

HGL#15

QVQLQESGGGLVQAGGSLRLSCAASGSIG **SMYVMS** WYRQAPGKEREPIA 49
ALMGSGSTTYADSVK GRFTISRDNEKNTMYLQMNSLTPEDTGVYYCAG 97
TGAEGHY WGQGTQVTVSS EPKTPKPQP 124

5

HGL#16

QVQLQESGGGLVQAGGSLRLSCAASGSDFR **YNAMA** WYRQAPGKQKRLVA 49
TITYTYRTNYADSVK GRFTISRDNARGTVYLMNSLKPEDTAVYYCAA 97
ARSLELTPTSVDY WGQGTQVTVSS EPKTPKPQP 130

10

4.2 Purification and characterisation of anti-Human Gastric Lipase V_HH fragments produced by *S. cerevisiae*.

15 From clones HGL#1, HGL#8, HGL#9, HGL#10, HGL#11 and
HGL#16 the V_HH encoding gene fragments were digested with
PstI and BstEII from the phage display vector pUR5071. The
DNA fragments were cloned into the episomal *S. cerevisiae*
plasmid pUR4547, which drives the secretion of V_HH domains
20 without any tags. In this way pUR5251, pUR5252, pUR5253,
pUR5254, pUR5255, pUR5256 were obtained encoding the V_HH
domains of clones HGL#1, HGL#8, HGL#9, HGL#10, HGL#11 and
HGL#16 respectively. The PstI/BstEII fragments were also
cloned into the episomal *S. cerevisiae* plasmid pUR4585,
25 which is responsible for the secretion of the V_HH domain
containing a myc- and a hexahistidine tag at its carboxy-
terminus. The clones coded pUR5257, pUR5258, pUR5259,
pUR5260, pUR5261 and pUR5262 were obtained containing the
V_HH encoding inserts of clones HGL#1, HGL#8, HGL#9, HGL#10,
30 HGL#11 and HGL#16 respectively.

Using 12 ml of culture supernatant from the induced
clones containing the hexahistidine tag the V_HH fragments

were purified with IMAC (according to the method described in paragraph 3.2). The yield was determined by measuring the optical density at 280 nm using the calculated molar extinction coefficient.

5

The efficiency of HGL recognition was determined for each individual antibody with ELISA using indirectly coated enzyme (Fig. 4A) and the degree of inhibition with the enzyme assay (Fig. 4B).

10

4.3 Measurement of inhibition in intestinal juices.

The measurement of the inhibition properties of the antibodies in accordance to the invention can be carried
15 out in accordance to the method described in Carriere et al
in Gastroenterology 1993: 105: 876-888.

14.03.2000

(25)

Claims

1. An antibody, or fragment thereof, capable of binding specifically to one or more human dietary enzymes, said antibody or fragment thereof comprising a heavy chain variable domain derived from an immunoglobulin naturally devoid of light chains, or a functional equivalent thereof.
2. An antibody, or fragment thereof, in accordance to claim 1, capable of binding specifically to one or more human lipases.
3. An antibody, of fragment thereof, in accordance to claim 2, capable of binding specifically to Human pancreatic lipase
4. An antibody, or fragment thereof, in accordance to claim 3, comprising 3 CDR regions, whereby CDR3 has one of the following sequences: DVRPYRTSRYLEX₃, QVRVRFSSDYTNV, LIRRKFTSEYNEY, LITRWDKSVNDY, RRSNYDRSWG DY, LISSYDGSWNDY, HITPAGSSNYVYGY or DIRKRFTSGYSHY, wherein X₃ is V or L or I.
5. An antibody, or fragment thereof, in accordance to claim 3 having one of the sequences HPL#11, HPL#12, HPL#13, HPL#14, HPL#15, HPL#18, HPL#19, HPL#22 or HPL#30.
6. An antibody, of fragment thereof, in accordance to claim 2, capable of binding specifically to Human gastric lipase.

7. An antibody, or fragment thereof, in accordance to claim 6, comprising 3 CDR regions, whereby CDR3 has one of the following sequences: ARSLX₁X₂TPTSYDY, RGGLTQYSEHDY, TGAEGHY, TDMGRYGTSEW; wherein X₁ is V or E and X₂ is Q or L.
8. An antibody, or fragment thereof, in accordance to claim 7 having one of the sequences HGL#1, HGL#4, HGL#8, HGL#9, HGL#10, HGL#11, HGL#15 or HGL#16.
9. Food product comprising an antibody or fragment thereof in accordance to one or more of the claims 1-8.
10. Pharmaceutical product comprising an antibody or fragment thereof in accordance to one or more of the claims 1-8
11. Use of an antibody or fragment thereof according to claim 1-8 in the preparation of a medicament or food for inhibiting the activity of one or more human dietary enzymes in the human body.
12. Use of a food product in accordance to claim 9 for the cosmetic control of body weight of human beings.

14. 03. 2000

(86)

Abstract

An antibody, or fragment thereof, capable of binding specifically to one or more human dietary enzymes, said antibody or fragment thereof comprising a heavy chain variable domain derived from an immunoglobulin naturally devoid of light chains, or a functional equivalent thereof.

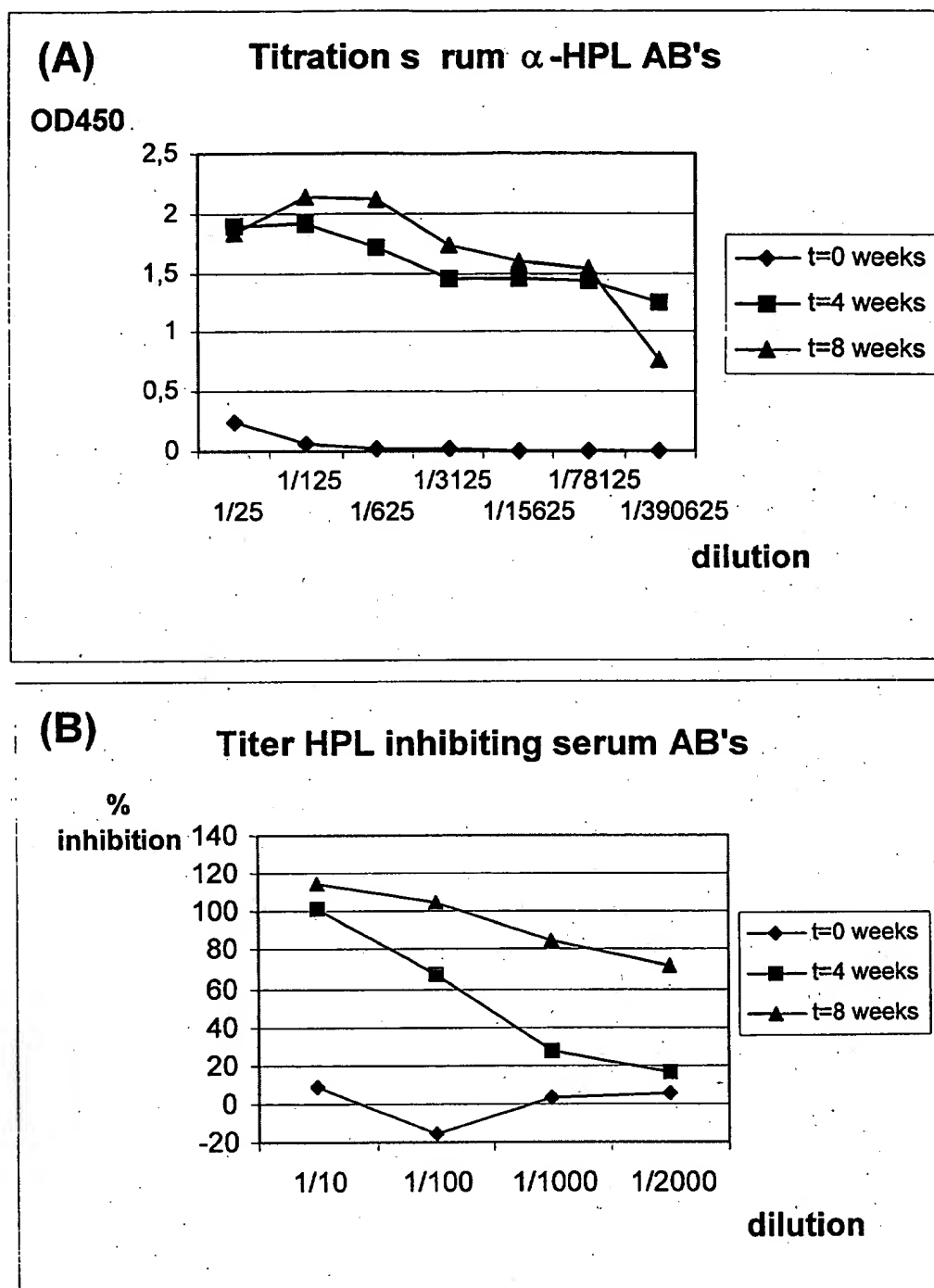


Figure 1

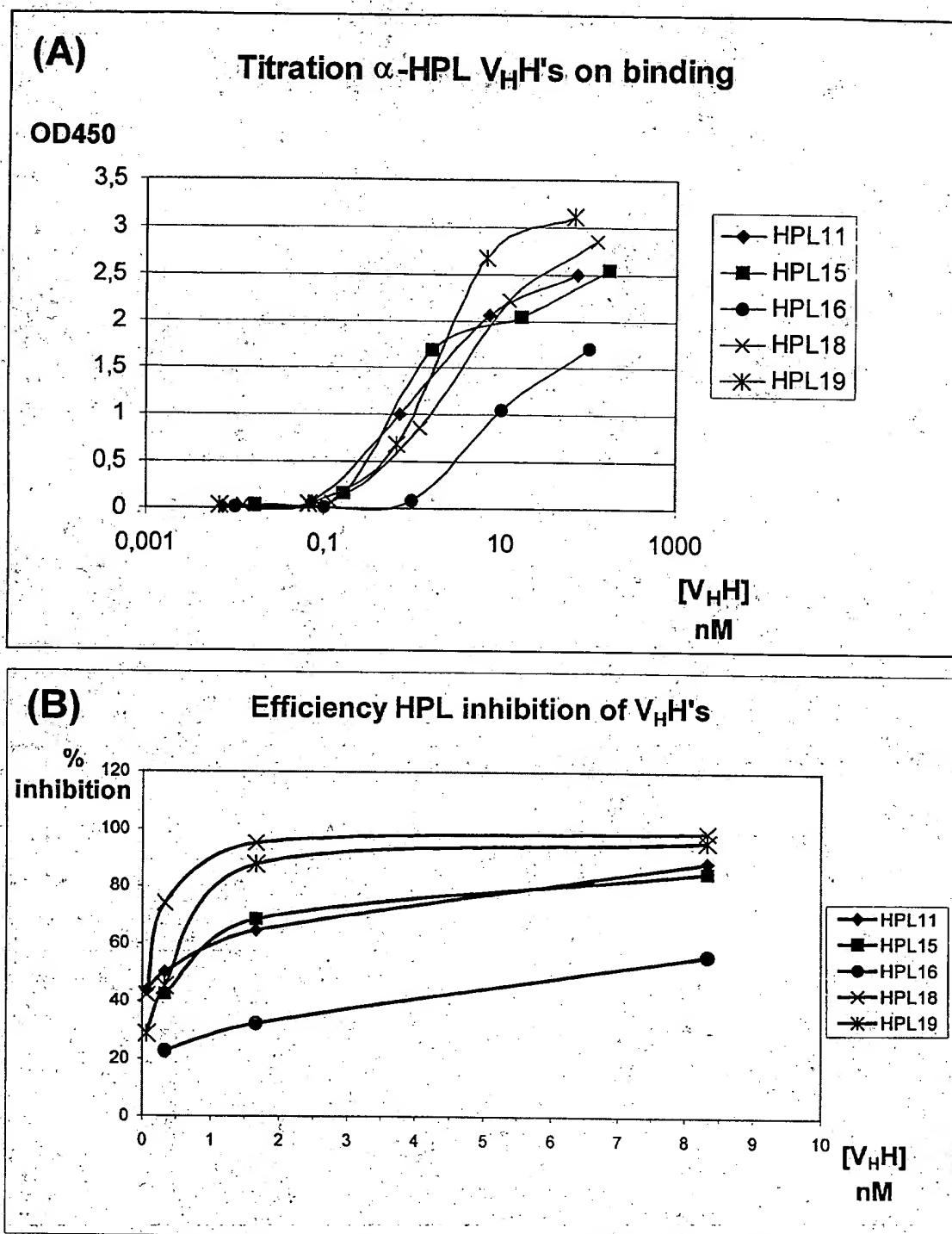
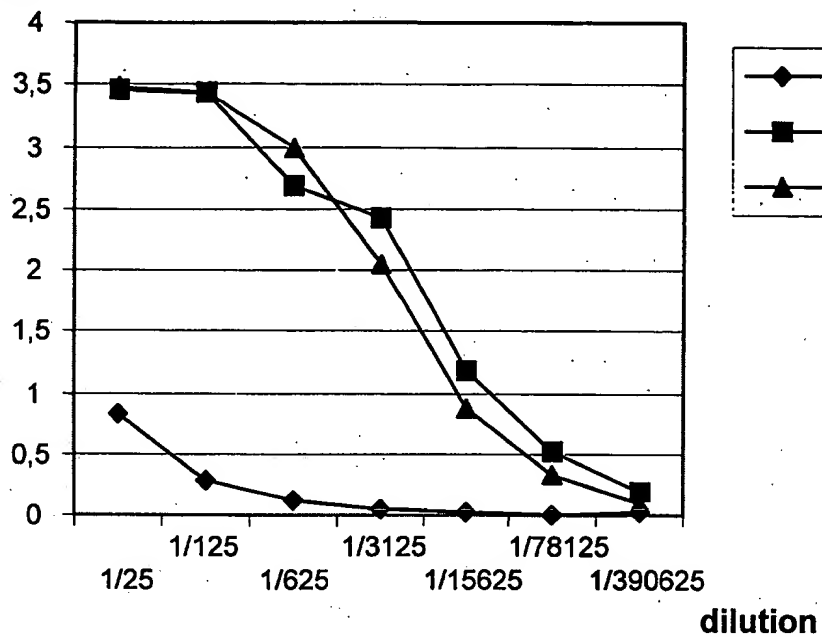


Figure 2

(A)

Titration serum α -HGL AB's

OD450



(B)

Titer inhibiting serum AB's

% inhibition

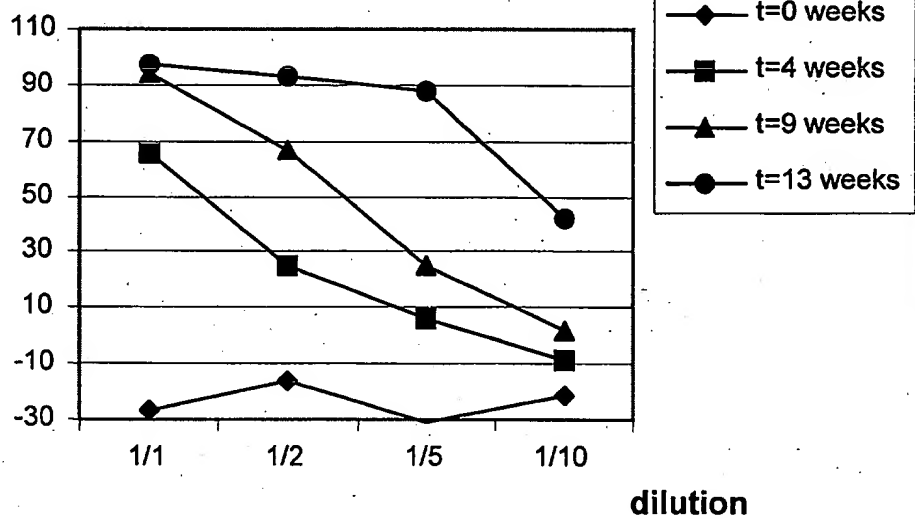


Figure 3

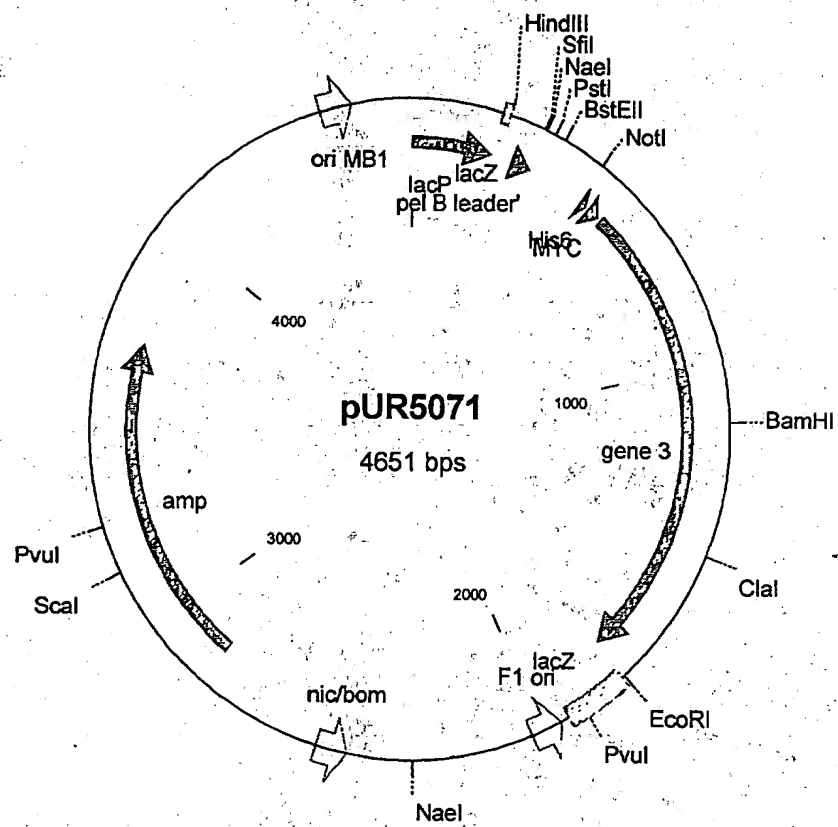


Figure 4